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TITLE: Method for preparing a pesticidally resistant rhizobium and agronomic composition thereof

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Nutritional Requirements of Rhizobia. In order to study the metabolism of these antifungal chemicals by the fungicide-tolerant strains, it was necessary to replace the complex, yeast extract-containing YEM medium with a simple mineral salts medium amended with only one or two growth factors.

Consequently, a modified Vincent's, supra, mineral salts medium containing the following macro- and micro-nutrients per liter of distilled water was prepared:

10.0 g mannitol, 1.0 g K₂HPO₄, 1.0 g KH₂PO₄, 1.0 g KNO₃, 0.2 g NaH₂PO₄.2H₂O, 0.18 g MgSO₄.0.13 g CaSO₄.2H₂O, 0.10 g Fe(NO₃)₂.9H₂O, 0.2 mg NaMoO₄.2H₂O, 0.2 mg ZnSO₄.7H₂O, 0.2 mg H₂BO₃, 0.2 mg MnSO₄.H₂O, 0.015 mg CuSO₄.5H₂O, and 0.001 mg Co(NO₃)₂.6H₂O. Portions (15.0 ml) of this mineral saltsmannitol medium were placed in 25 ml test tubes, and 7 of these tubes were amended with 1.3 mg/l of each of the following seven vitamins: p-aminobenzoic acid, pyridoxine phosphate, inositol(meso), thiamine mononitrate, calcium pantothenate, riboflavin, and biotin. The growth factors were obtained from Nutritional Biochemicals Corp., Cleveland, Ohio. Each tube was then inoculated

with 0.1 ml of a cell suspension derived from a 48-hour culture of the rhizobia that had been washed three times with 0.1 M phosphate buffer (pH 7.0). The cell density was 0.10 as measured by a spectrophotometer, model Spectronic 20, at a wavelength of 540 nm. Controls consisting of inoculated and uninoculated mineral salts-mannitol media were also prepared. Each treatment was in duplicate. All the tubes were incubated on a rotary shaker at 30.degree. C. for two days, with a shaker speed of 180 rpm. Growth of bacteria in each tube was measured turbidimetrically at intervals of three hours using a spectrophotometer (Spectronic 20), and measuring optical density at 540 nm. As shown in Table 8, the adapted cowpea Rhizobium strain KO4SRPR grew well in a mineral salts-mannitol medium amended with calcium pantothenate, whereas growth was somewhat slower in the same medium treated with other growth factors or with no vitamins. Thiram-tolerant *R. meliloti* 87TR grew readily in the mineral salts-mannitol broth amended with inositol(meso) or pyridoxine phosphate, while spergon-resistant *R. phaseoli* 203CR grew reasonably well when the medium was treated with either calcium pantothenate or thiamine mononitrate. Growth turbidity was observed visually in the case of *R. meliloti* 87TR and *R. phaseoli* 203CR.

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TITLE: Nitrogen fixation regulator genes

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Despite the ability of certain plants to induce nitrogenase activity in a symbiotic relationship with some species of Rhizobium, the genetic analysis of biological nitrogen fixation has previously been confined to free living nitrogen fixing organisms, in particular Klebsiella pneumoniae. There are 17 linked nitrogen fixation (nif) genes arranged in at least 7 transcriptional units in the nif cluster of Klebsiella (Kennedy, C., Cannon, F., Cannon, M., Dixon, R., Hill, S., Jensen, J., Kumar, S., McLean, P., Merrick, M., Robson, R. and Postgate, J. (1981) In Current Perspectives in Nitrogen Fixation (A. H. Gibson, W. E. Newton, eds.) Canberra: Australian Academy of Science, pp. 146-156; and Reidel, G. E., Ausubel, F. M. and F. M. Cannon (1979) Proc. Natl. Acad. Sci. U.S.A. 76: 2866-2870). Three of these genes, nifH, nifD and nifK encode the structural proteins of the nitrogenase enzyme complex (viz. the Fe-protein subunit (dinitrogenase reductase) and the .alpha.- and .beta.-subunits of the Mo-Fe protein (dinitrogenase) respectively. Dinitrogenase is an .alpha..sub.2 .beta..sub.2 tetramer in which the two non-identical .alpha. and .beta. subunits have similar molecular weights of 55,000 to 60,000. Dinitrogenase reductase is a dimer of two identical subunits each having a molecular weight around 35,000. These genes are linked on the

same operon in *K. pneumoniae* and are transcribed from a promoter adjacent to the *nifH* gene. A similar situation (*nifHDK*) was found in two fast-growing *Rhizobia*, *R. meliloti* (Ruvkun, G. B., et al. (1982) *Cell* 29: 551-559) and *R. leguminosarum* (Schetgens, T. M. P. et al. (1984) Identification and analysis of the expression of *Rhizobium leguminosarum* PRE symbiotic genes, p. 699, In C. Veeger and W. E. Newton (eds.) *Advances in nitrogen fixation research*. Martinus Nijhoff/Dr. W. Junk Publishers, The Hague). In the slow-growing *R. japonicum*, it has been found that *nifDK* forms one operon and that *nifH* is located elsewhere on the genome (Fuhrmann, M. and H. Hennecke (1982) *Mol. Gen. Genet.* 187: 419-425). A similar observation was made with another member of the slow-growing rhizobia, *Rhizobium* sp. *Parasponia*: a *nifH* region was found not to be linked to *nifD* (Scott, K. F., et al. (1983) *DNA* 2: 141-148). Yet a different arrangement was detected in the cyanobacterium *Anabaena* sp. 7120, in which *nifHD* is separated from *nifK* (Rice, D., et al. (1982) *J. Biol. Chem.* 257: 13157-13163). The remainder of symbiotic genes contain information required for bacterial attachment, root hair curling, initiation and development of nodules and establishment of symbiotic relationships. In addition, regulatory sequences such as promoters, operators, attenuators, and ribosome binding sites are found adjacent to the coding regions. These regulatory sequences control the expression of the structural genes, i.e., the coding sequences downstream in the 3'-direction of the DNA reading strand.